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Characteristics of IL-6 and TNF-α Production by Respiratory Syncytial Virus-Infected Macrophages in the Neonate

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The production of IL-6 and TNF-α and the expression of their mRNA were studied with neonatal (cord blood) and adult blood monocyte-derived macrophages (MDM) after in vitro infection with respiratory syncytial virus (RSV). Cord blood MDM exhibited production of high levels of IL-6 within 24 hr after infection. Little or no IL-6 production was detected after 24–48 hr and after in vitro stimulation with inactivated (nonreplicating) virus. Adult blood MDM also produced high levels of IL-6 within 24 hr of RSV infection. Unlike cord blood MDM, adult MDM demonstrated significant activity of IL-6 after 24 hr of infection with live RSV and after exposure to the inactivated virus. The pattern of TNF-α production by cord and adult blood MDM after live RSV infection resembled closely the pattern of IL-6 production. Both cell types produced TNF-α in the first 24 hr after infection. However, little or no production was observed after 24 hr of infection and after exposure to the inactivated virus. The profile of mRNA expression was similar to the production of IL-6 or TNF-α. mRNA expression occurred over a shorter period in cord blood MDM. These observations suggest that inflammatory and immunoregulatory cytokines, such as IL-6 and TNF-α, are produced by neonatal as well as previously primed adult macrophages. However, neonatal cells may be less efficient in inducing IL-6 production.

KEY WORDS: cytokine, IL-6, TNF-α, mRNA, monocyte-derived macrophages

INTRODUCTION

Respiratory syncytial virus (RSV) causes symptoms that are usually restricted to the upper respiratory tract in adults and older children, whereas young infants develop frequently bronchiolitis or pneumonia, often despite the presence of maternally transferred specific antibody [Parrott et al., 1973; McIntosh and Chanock, 1990; Pringle, 1987]. Previous studies have identified lower serum and secretory antibody responses to RSV in young infants than in older infants and children, and the immaturity of the immune system has been proposed as one of the reasons for the decreased virus-specific response [Richardson et al., 1978; McIntosh et al., 1978; Kaul et al., 1981; Murphy et al., 1986a,b; Yamazaki et al., 1994].

Alveolar macrophages have been reported to be a possible target for RSV infection [Morahan et al., 1985; Panuska et al., 1990a]. A rapid response by macrophages as manifested by production of a wide range of immunological mediators including TNF-α, IL-1, IL-6, IL-8, and prostaglandin E_2 has been observed after infection with RSV and macrophages appear to have a prominent role in the initiation of the immune response [Roberts et al., 1986; Panuska et al., 1990b; Becker et al., 1991]. The in vitro studies cited above have utilized macrophages or monocytes derived from either normal adults or adults with RSV infection. IL-6 and TNF-α production in the mouse model inoculated with RSV was also examined [Hayes et al., 1994]. However, the mechanisms underlying production of such cytokines in macrophages of young infants with RSV infection have not yet been defined, partly because of the difficulty in getting alveolar macrophages from young infants.

In this study, the characteristics of induction of major immunoactive or antiviral cytokines, IL-6 and TNF-α, were examined in macrophages of young infants in response to RSV infection, by infecting monocyte-derived macrophages (MDM) obtained from normal neonates (cord blood) with RSV.

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MATERIALS AND METHODS

Isolation and Culture of Human Monocyte/Macrophages

Heparinized cord blood and peripheral venous blood samples obtained from six healthy neonates and six healthy adults were sedimented over Ficoll-Hypaque (Pharmacia). Mononuclear cells were collected and washed three times with RPMI 1640 medium. The cell concentration was adjusted to 2×10^7 cells/ml in RPMI 1640 supplemented with 20% human AB serum. Three hundred microliters of samples (6×10^6 cells) were applied to each well of 24 well semimicro plates (Nunc, Denmark) which had been precoated with human AB serum for 1 hr at 37°C and incubated 2 hr in 5% CO_2. The plates were washed twice with RPMI 1640 medium to remove nonadherent cells. Over 90% of adherent cells were positive for nonspecific esterase. They were cultured for 2 days in RPMI 1640 with 20% human AB serum and another 4 days in RPMI 1640 medium with 20% heat-inactivated fetal calf serum (FCS).

Virus Strains

RSV long strain (prototype RSV group A strain) was grown in HEp-2 cells, and the culture fluid was centrifuged (1,500*g*, 10 min) and preserved at −70°C. The titer of stock virus was 2×10^7 plaque-forming unit (pfu)/ml. Aliquots of the stock virus preparation was placed in a flask 10 cm from ultraviolet (UV) light and irradiated for 20 min to inactivate the virus. Complete inactivation of virus was confirmed by plaque formation of UV-treated RSV preparation (data not shown). Uninfected HEp-2 cell culture fluid was processed similarly for use as controls.

Exposure of Cells to RSV

Six days after isolation, MDM were washed two times in RPMI 1640 and inoculated with 150 μl of stock virus (3×10^6 pfu) for 1 hr at 37°C. The same volumes of UV-inactivated virus or HEp-2 cell preparations were used similarly. Cells were then washed with RPMI two times and incubated in 0.7 ml RPMI 1640 with 20% FCS. Culture media were collected at 24 hr after infection and reincubated with fresh medium for another 24 hr; aliquots of medium were then harvested. The MDM in 24 wells were fixed for 15 min at 4°C in 80% (v/v) acetone-phosphate-buffered saline (PBS) [Anderson et al., 1985] at 24 and 48 hr after infection and the percentage of infected cells was measured by indirect immunofluorescent antibody testing with anti-RSV rabbit antibodies (DAKOPATTS, Denmark). The titers of infectious virus in culture media harvested at 24 and 48 hr after infection were determined by plaque formation.

For expression of mRNA for IL-6, TNF-α, and β-actin, MDM were harvested 2, 6, 12, and 24 hr after RSV infection; MDM were washed with PBS three times and treated with 0.8 ml of RNAzol B (Biotecx Laboratories, Houston, TX) for RNA extraction.

Cytokine Assay

The presence of IL-6 and TNF-α in the MDM culture supernatants harvested at 24 and 48 hr after infection was measured by means of quantitative enzyme-linked immunosorbent assays (ELISAs; Quantikine; R and D systems, Minneapolis, MN). Samples were diluted five times with dilutants. The detection limits for ELISAs for the demonstration of human IL-6 and TNF-α were 60 and 80 pg/ml of culture fluid, respectively (manufacturer's information).

For the purpose of calculation of mean titers in each group, specimens with cytokine levels under the limit of detection were given a value of half of limit of detection. Statistical comparison between groups was made using the Student's t test.

Analysis of mRNA of Cytokine

The total cellular RNA was isolated from MDM using RNAzol B and tested for mRNA of IL-6 and TNF-α, employing specific reverse transcriptase polymerase chain reaction (RT-PCR) assays. For internal control, β-actin mRNA was also amplified by RT-PCR. Fifty nanograms of the RNA were used for RT-PCR. For cDNA synthesis, 40 μl RNA solution (50ng) and 150 pg/3 μl random hexmer (Takara, Kyoto, Japan) were heated at 70°C for 10 min and cooled rapidly. After adding 18 μl of 5 × first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 10 mM of DTT (GIBCO BRL, Gaithersburg, MD). 0.5 mM of dNTP (Takara), and 200 units of M-MLV reverse transcriptase (GIBCO BRL), the mixture was stored at 37°C for 1 hr. The specific primer pairs were synthesized according to the sequences described previously [Becker et al., 1991; Okamoto et al., 1993]. The PCR reaction mixture contained 50 ng of cDNA, 10 μl of 10 × PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 8 μl of 2.5 mM dNTP (Takara), 100 pM 5′ and 3′ primers, and DEPC water for a total volume to 100 μl. After denaturing at 94°C for 5 min and cooling rapidly, the mixture was seeded with 2.5 U of thermostable Taq polymerase (Takara), and 27 cycles of amplification were carried out. Each cycle consisted of warming at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Finally they were incubated at 72°C for 10 min. Ten microliter samples of the products were analyzed by electrophoresis of 3% agarose gel, and the amplified product was visualized by UV fluorescence after staining with ethidium bromide. A 1 Kb DNA Ladder (GIBCO BRL) was run in parallel as a molecular weight marker. The specificity of the PCR products for cytokines was confirmed by their predicted size on agarose gels [Becker et al., 1991; Okamoto et al., 1993]. The specificity of the PCR products was also validated by dot-blot analysis using ^{32}P end-labeled internal oligonucleotide probes as described previously [Okamoto et al., 1993] (data not shown).

TABLE I. Titer of Infectious Virus Recovered From Cord and Adult Blood MDM at 24 and 48 Hr After Infection†

	Virus titer ($\times 10^3$ pfu/ml)			
	24 Hr		48 Hr	
Cord blood MDM (n = 6)	3.2 ± 0.5	*	0.8 ± 0.2	**
Adult blood MDM (n = 6)	2.8 ± 0.2		1.8 ± 0.2	

†Results are presented as mean and standard error of the mean.
*Student's t test (unpaired); not significant.
**Student's t test (unpaired); $P < 0.02$.

RESULTS

Susceptibility of MDM to Infectious RSV

Infection with RSV induced a cytopathic effect (CPE), which was manifested by rounding of the cells in both MDM from cord and adult blood as early as 24 hr after infection. There was no apparent change of remaining cells in the MDM cultures 48 hr after infection. Around 80% of the cord and adult blood MDM were positive for viral antigen 24 and 48 hr after infection, as determined by immunofluorescent antibody staining. There was no significant difference in the percentage of cells infected between cord and adult blood MDM (data not shown).

The titers of infectious virus recovered from cord and adult blood MDM 24 hr after infection were also similar ($3.2 \pm 0.5 \times 10^3$ and $2.8 \pm 0.2 \times 10^3$ pfu/ml; M ± SE, $P > 0.6$). The titers of infectious virus produced by both cell types decreased 48 hr after infection. However, the amount produced by cord blood MDM 48 hr after infection ($0.8 \pm 0.2 \times 10^3$ pfu/ml) was significantly lower than that observed in adult blood MDM ($1.8 \pm 0.2 \times 10^3$ pfu/ml; M ± SE, $P < 0.02$; Table I).

Cytokine Secretion by RSV-Infected MDM

Culture fluids of RSV-infected MDM incubated for 0–24 hr and 24–48 hr after infection were harvested and assayed for IL-6 and TNF-α activity and compared with MDM exposed to UV-inactivated RSV and nonviral control (HEp-2 cell) antigens (Fig. 1). Cord blood MDM produced high levels of IL-6 (4,383 ± 765 pg/ml) and similar levels of IL-6 were detected in adult blood MDM (4,117 ± 659 pg/ml; M ± SE, $P > 0.4$) for the first 24 hr after infection. Subsequently, however, IL-6 production by cord blood MDM (600 ± 160 pg/ml) was somewhat lower compared to the levels in adult blood MDM (1,421 ± 277 pg/ml; M ± SE, $P < 0.05$). On the other hand, the levels of IL-6 produced by cord blood MDM exposed to inactivated virus for the first 24 hr (571 ± 98 pg/ml) were significantly lower than those observed in adult blood MDM exposed to the inactivated virus (1,960 ± 327 pg/ml; M ± SE, $P < 0.005$).

Cord and adult blood MDM produced moderate levels of TNF-α for the first 24 hr after infection (1,436 ± 570 pg/ml and 1,350 ± 275 pg/ml; M ± SE, $P > 0.6$). After 24 hr, both cord and adult blood MDM infected with RSV ceased production of TNF-α. No significant increase in TNF-α occurred in MDM exposed to the inactivated virus.

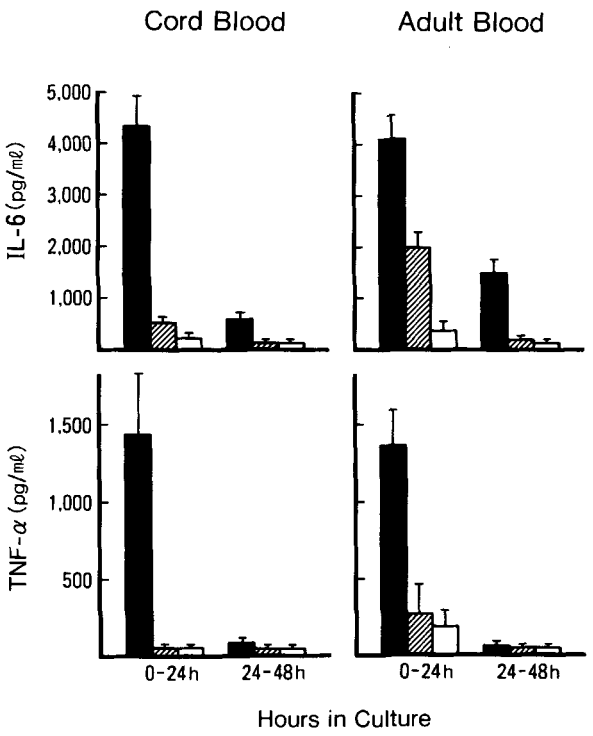

Fig. 1. IL-6 and TNF- production by cord and adult blood MDM exposed to live and inactivated RSV. Supernatants were collected at 24-hr intervals and reincubated with fresh medium. Supernatants of MDM exposed to live RSV (first bar; ■), inactivated RSV (second bar; ▨), and HEp-2 cell control (third bar; □) were tested. Data represent mean + SE in each six experiments.

Time Course of Cytokine mRNA Induction in RSV-Infected MDM

The kinetics of expression for the mRNA of IL-6 and TNF-α was determined in MDM infected with RSV. Figure 2 shows the representative data of the ethidium bromide-stained PCR products of mRNA of cord and adult blood MDM in the agarose gel. β-actin mRNA was included as an internal control. The IL-6 and TNF-α mRNA in cord blood MDM appeared within 2 hr after infection and continued to be expressed until 6 hr after infection. Cord blood MDM exposed to inactivated RSV did not express IL-6 or TNF-α mRNA at any time during the 24 hr observation period. On the other hand, adult blood MDM infected with live RSV expressed both IL-6 and TNF-α mRNA within 2–12 hr after infection. Adult blood MDM exposed to inactivated RSV expressed IL-6 mRNA at only 2 hr after infection.

DISCUSSION

This study employed cord blood MDM and an in vitro RSV infection model to explore the capacity of neonatal macrophage to produce inflammatory cytokines following RSV infection. In earlier studies, alveolar macrophages have been shown to be largely derived from blood monocytes [Thomas et al., 1976], and previous

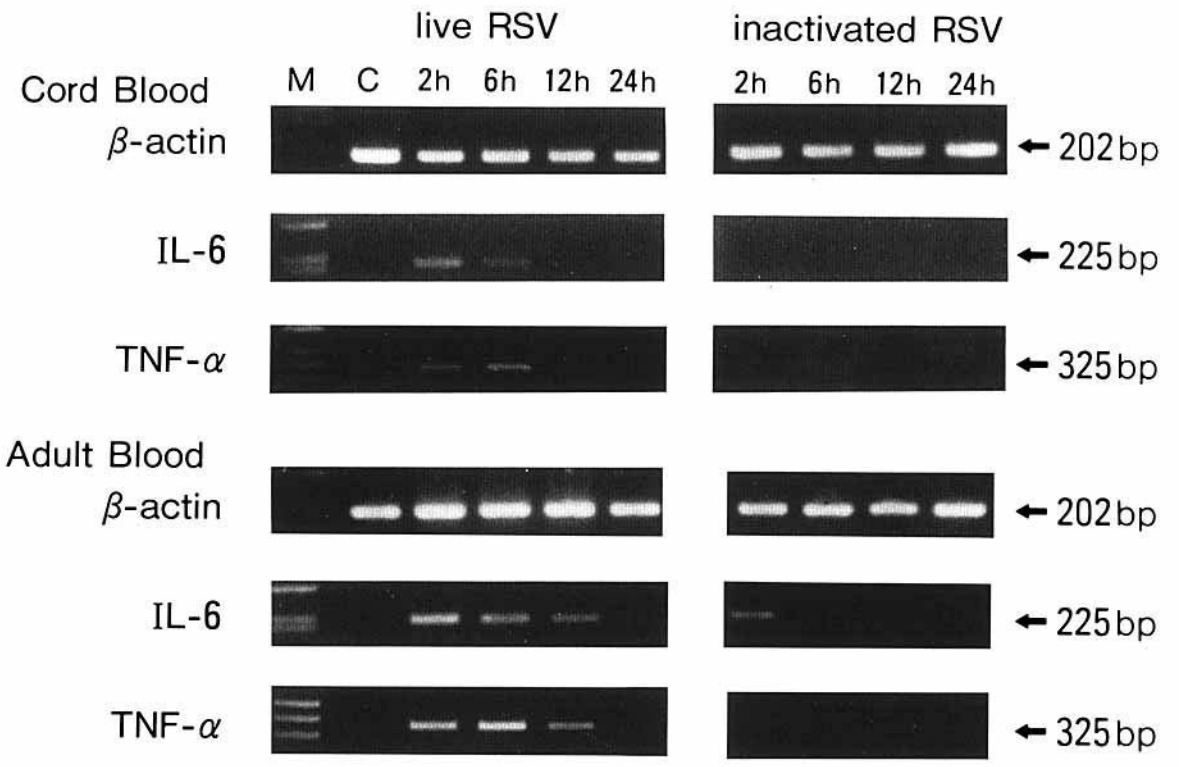

Fig. 2. Time course of cytokine mRNA (β-actin, IL-6, and TNF-α) expression in cord and adult blood MDM exposed to live and inactivated RSV determined by RT-PCR assays. All mRNA expression was determined at 27 cycles. **Lane M:** Marker (1 Kb DNA Ladder); **lane C:** untreated MDM. Cytokine mRNA expression was assessed after indicated time of treatment.

studies have suggested that monocytes might mature into macrophages upon in vitro cultivation [Mayernik et al., 1983].

This is the first analysis of mRNA expression and induction of two inflammatory cytokines, IL-6 and TNF-α, in RSV-infected monocytes/macrophages of infants. Becker et al. [1991] have shown immediate increases in production of these cytokines from RSV-infected alveolar macrophages from normal adults. Middula et al. [1993] observed the expression of IL-1 and TNF-α in alveolar macrophages from infants with RSV infection using fluorescent antibody staining. The present study has shown that the macrophages in neonates can produce IL-6 and TNF-α to the same degree as adult macrophages, in response to RSV infection.

However, some differences were found between cord and adult blood MDM for induction of IL-6. Production of IL-6 by RSV-infected cord blood MDM lasted for a shorter period than that observed for adult blood MDM. These differences correlated roughly to the duration of mRNA expression. There was no apparent difference in the percentage of infected cells between cord and adult MDM at both 24 and 48 hr after infection. On the other hand, the new virus titer produced by cord blood MDM after the second 24 hr was apparently lower than that from adult MDM. The shorter duration of IL-6 induction with RSV infection observed in cord blood MDM may reflect the more rapid death of neonatal cells than observed in adult blood MDM.

Of particular importance is the observation of lower IL-6 induction in response to inactivated virus in cord blood MDM, which was also supported by mRNA data and appeared to reflect the events at the transcriptional level. Adult blood MDM produced moderate IL-6 after treatment with inactivated virus, as observed by Becker et al. [1991]. It is possible that alveolar macrophages of young infants might not be able to produce as much IL-6 during the processing phase of uninfectious RSV particles as adult macrophages. During the course of RSV replication in infants, some uninfectious RSV should be produced possibly by inactivation by preexisting (maternal) antibodies or nonspecific mucosal defense mechanisms.

It has been shown that IL-6 plays an important role in mucosal IgA antibody responses in the mouse model [Beagley et al., 1989; Ramsay et al., 1994]. The diminished capacity of cord blood cells to synthesize IL-6 may be related to the immature secretory immune functions in young infants. The present in vitro results might suggest that inhaled live and attenuated RSV vaccines are more effective than the inhaled nonreplicating subunit or inactivated RSV antigens for mounting an effective secretory immune response in young infants [Crowe et al., 1994; Oien et al., 1994].

REFERENCES

Anderson LJ, Hierholzer JC, Bingham PG, Stone YO (1985): Microneutralization test for respiratory syncytial virus based on an enzyme immunoassay. Journal of Clinical Microbiology 22:1050–1052.

Beagley KW, Eldridge JH, Lee F, Kiyono H, Everson MP, Koopman WJ, Hirano T, Kishimoto T, McGhee JR (1989): Interleukins and IgA synthesis: Human and murine interleukin 6 induce high IgA secretion in IgA-committed B cells. Journal of Experimental Medicine 169:2133–2148.

Becker S, Quay J, Soukup J (1991): Cytokine (tumor necrosis factor, IL-6 and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. Journal of Immunology 147:4307–4312.

Crowe JE Jr, Bui PT, London WT, Davis AR, Hung PP, Chanock RM, Murphy BR (1994): Satisfactorily attenuated and protective mutants derived from a partially attenuated cold-passaged respiratory syncytial virus mutants by introduction of additional attenuating mutations during chemical mutagenesis. Vaccine 12:691–699.

Hayes PJ, Scott R, Wheeler J (1994): In vivo production of tumour necrosis factor-α and interleukin-6 in Balb/c mice inoculated intranasally with a high dose of respiratory syncytial virus. Journal of Medical Virology 42:323–329.

Kaul TN, Welliver RC, Wong DT, Udwadia RA, Riddlesberger K, Ogra PL (1981): Secretory antibody response to primary and secondary infection with respiratory syncytial virus infection. American Journal Diseases of Children 135:1013–1016.

Mayernik DG, Ul-haq A, Rinehart JJ (1983): Differentiation-associated alteration in human monocyte-macrophages accessory cell function. Journal of Immunology 130:2156–2160.

McIntosh K, Chanock RM (1990): Respiratory syncytial virus. In Fields BN, Knipe DM (eds): "Fields' Virology." New York: Raven Press, pp 1045–1072.

McIntosh K, Masters HB, Orr I, Chao RK, Barkin RM (1978): The immunologic response to infection with respiratory syncytial virus in infants. Journal of Infectious Diseases 138:24–32.

Middula F, Villani A, Panuska JR, Dab I, Kolls JK, Merolla R, Ronchetti R (1993): Respiratory syncytial virus lung infection in infants: Immunoregulatory role of infected macrophages. Journal of Infectious Diseases 168:1515–1519.

Morahan PS, Connor JR, Leary KR (1985): Viruses and the versatile macrophages. British Medical Bulletin 41:15–21.

Murphy BR, Alling DW, Snyder MH, Walsh EE, Prince GA, Chanock RM, Hemming VG, Rodriguez WL, Kim HW, Graham BS, Wright PF (1986a): Effect of age and pre-existing antibody of serum antibody response of infants and children to the F and G glycoproteins during respiratory syncytial virus infection. Journal of Clinical Microbiology 24:894–898.

Murphy BR, Graham BS, Prince GA, Walsh EE, Chanock RM, Karzon DT, Wright PF (1986b): Serum and nasal-wash immunoglobulin G and A antibody response of infants and children to respiratory syncytial virus F and G glycoproteins following primary infection. Journal of Clinical Microbiology 23:1009–1014.

Oien NL, Brideau RJ, Walsh EE, Wathen MW (1994): Induction of local and systemic immunity against human respiratory syncytial virus using a chimeric FG glycoprotein and cholera toxin B subunit. Vaccine 12:731–735.

Okamoto Y, Kudo K, Ishizawa K, Ito E, Togawa K, Saito I, Moro I, Patel JA, Ogra PL (1993): Presence of respiratory syncytial virus genomic sequences in middle ear fluid and its relationship to expression of cytokines and cell adhesion molecules. Journal of Infectious Diseases 168:1277–1281.

Panuska JR, Cirino N, Midulla F, Despot JE, McFadden ER, Huang YT (1990a): Productive infection of isolated human alveolar macrophages by respiratory syncytial virus. Journal of Clinical Investigation 86:113–119.

Panuska JR, Midulla F, Cirino N, Villani A, Gilbert IA, McFadden ER, Huang YT (1990b): Virus induced alteration in macrophage production of tumor necrosis factor and prostaglandin E2. American Journal of Physiology 259:L396–L402.

Parrott RH, Kim HW, Arrobio JO, Hodes DS, Murphy BR, Brandt CD, Camargo E, Chanock RM (1973): Epidemiology of respiratory syncytial virus infection in Washington D.C. II. Infection and disease with respect to age, immunologic status, race and sex. American Journal of Epidemiology 98:289–300.

Pringle BC (1987): Paramyxoviruses and diseases. In Russell WC, Almond JW (eds): "Molecular Basis of Virus Disease." Cambridge: Cambridge University Press, pp 51–90.

Ramsay AJ, Husband AJ, Ramshaw IA, Bao S, Matthaei KI, Koehler G, Kopf M (1994): The role of interleukin-6 in mucosal IgA antibody responses in vivo. Science 264:561–563.

Richardson LS, Yolken RH, Belshe RB, Camargo E, Kim HW, Chanock RM (1978): Enzyme-linked immunosorbent assay for measurement of serological response to respiratory syncytial virus infection. Infection and Immunity 20:660–664.

Roberts NJ, Prill AH, Mann TN (1986): Interleukion 1 and interleukin 1 inhibitor production by human macrophages exposed to influenza virus or respiratory syncytial virus. Journal of Experimental Medicine 163:511–519.

Thomas ED, Ramberg RE, Sale GE, Sparkes RS, Golde DW (1976): Direct evidence for a bone marrow origin of the alveolar macrophages in man. Science 192:1016–1018.

Yamazaki H, Tsutsumi H, Matsuda K, Nagai K, Ogra PL, Chiba S (1994): Effect of maternal antibody on IgA antibody response in nasopharyngeal secretion in infants and children during primary respiratory syncytial virus infection. Journal of General Virology 75:2115–2119.